

ORIENTATION OF HYDROGENASE IN THE PLASMA MEMBRANE OF *PARACOCCLUS DENITRIFICANS*

J. DOUSSIÈRE, F. PORTE and P. M. VIGNAIS

Biochimie/DRF, (INSERM U.191 et ER CNRS 235) CENG 85-X, 38041 Grenoble Cédex, France

Received 31 March 1980

1. Introduction

Autotrophic growth on hydrogen induces the synthesis of hydrogenase in *Paracoccus denitrificans* [1]. The hydrogenase is an intrinsic membrane-protein which is part of the respiratory chain in the plasma membrane of *P. denitrificans* [2–5]. Protons are ejected from cells of *P. denitrificans* during oxidation of endogenous substrates [6]. Similarly, hydrogenase-containing spheroplasts of *P. denitrificans* released protons in the outward direction when oxidizing H₂ by O₂ [7].

The question arose whether the hydrogenase per se could act as a loop in the Mitchelian sense [8], i.e., could create an electrochemical gradient of protons, positive outside, which could be used by the ATPase, cytoplasmically oriented, to make ATP.

Using hydrogenase-containing membrane vesicles from *Escherichia coli* [9] it was concluded that an outward ejection of protons was linked to the oxidation of hydrogen by fumarate. However, from the data in [9] it is not possible to know whether or not the electron pathway from H₂ to fumarate involved a respiratory 'loop'.

The low potential electron acceptor, benzylviologen, can reoxidize H₂-reduced hydrogenases [4,10] and react directly with the membrane-bound hydrogenase of *P. denitrificans* even in whole cells [4]. It was therefore chosen as oxidant to measure the direction of proton ejection during reoxidation of the H₂-reduced membrane-bound hydrogenase of *P. denitrificans*.

Abbreviations: benzylviologen, *N,N'*-dibenzyl-4,4'-dipyridyl-dihydrochloride; DCPIP, 2,6 dichloroindophenol; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazine; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid; MES, 4-morpholino-ethanesulphonic acid

This paper describes the method used to measure directly the protons translocated across the plasma membrane of spheroplasts of *P. denitrificans* when H₂-reduced hydrogenase was being reoxidized by benzylviologen. It shows that the protons were released inside the spheroplasts and that this oxidation did not lead to ATP synthesis.

2. Materials and methods

Bacterial culture (*Paracoccus denitrificans*, DSM 65 strain 381, a gift from Professor H. G. Schlegel, Inst. Microbiol., Göttingen) and preparation of spheroplasts were as in [4] except that the spheroplasts were washed and resuspended in 0.3 M sucrose, 0.1 M KCl, 50 μM MES, (pH 6.5). Membrane particles were prepared by sonication of cells in 0.15 M KCl, 4 times for 30 s, with a Branson Sonifier at maximum output. The suspension was then centrifuged at 6500 × *g* for 10 min to remove intact cells and cell debris. The pellet was treated again by sonication, and centrifuged at low speed. The pooled supernatant fluids were centrifuged at 100 000 × *g* for 1 h. This last pellet of membrane vesicles was resuspended in 0.15 M KCl.

The pH measurements were made with an air-tight titration assembly TTA 60 (Radiometer): the chamber contained 3 ml medium and was maintained under anaerobiosis by a stream of argon. The pH electrodes (calomel electrode K 4040, and glass electrode G 2040 C-Radiometer) were connected through a rapidly responding pH meter PHM 64 (Radiometer) to a pen recorder (SEFRAM). The sensitivity and effective range of the measurement of the pH change was increased by mounting, 'in opposition', a second

pH meter with a second glass electrode dipped in the chosen reference buffer.

ATPase activity was measured by monitoring phosphate [11] release in an assay medium containing 10 mM KCl, 6 mM $MgCl_2$, 150 mM sodium bicarbonate, 100 mM Tris-MES buffer (pH 8), 10 mM ATP and 1 mg protein in 1 ml total vol. The incubation medium was supplemented with 0.5 M sucrose when spheroplasts were used.

NADH-dichlorophenolindophenol oxidoreductase was measured as in [12].

The adenine-nucleotide content of whole cells was determined in the perchloric acid extract after neutralization with KOH. ATP, ADP and AMP were estimated as in [13].

FCCP was obtained from Pierce (Rockford, IL); benzylviologen and DCPIP from Serva (Heidelberg); HEPES, MES and bee venom from Sigma Chemical Co., (St Louis, MO).

3. Results and discussion

3.1. Orientation of membrane vesicles

Proton ejection was measured with two types of particles; spheroplasts and sonicated vesicles. Spheroplasts, which were obtained after lysozyme digestion of the cell wall, had the plasma membrane in the same orientation as the whole cells. Removal of the cell wall allowed an easier interaction of chemicals, such as benzylviologen or the uncoupler FCCP, with the plasma membrane.

Membrane vesicles were obtained by sonication of whole cells. This treatment inverts the membrane. However, it is necessary to assess the percentage of inverted and right-side out vesicles. The orientation of the membrane in sonicated vesicles was estimated from the activity of two marker enzymes, the NADH-DCPIP oxidoreductase [12] and the ATPase [5,7]. These activities were, for NADH-DCPIP oxidoreductase 16 and 71 nmol NADH reoxidized $\cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ in spheroplasts and in membrane vesicles, respectively, and for ATPase 5 and 68 nmol ATP hydrolyzed $\cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ in spheroplasts and in membrane vesicles, respectively. The much higher activity in membrane vesicles indicates that the substrates (NADH or ATP) had much better access to the enzymes than in spheroplasts. Consequently when the permeability barrier was decreased by treatment with bee venom [12] or with Triton

X-100 the increase in activity of the two marker enzymes was higher with spheroplasts (3–4-fold) than with membrane vesicles (30–40%). These data showed that while in spheroplasts the plasma membrane had the same orientation as in whole cells, the sonicated membrane vesicles were mainly (70–80%) inside-out vesicles.

3.2. Hydrogen-dependent release of protons in spheroplasts

3.2.1. Inward proton release with benzylviologen

Benzylviologen had been shown [4] to accept electrons from hydrogen through the hydrogenase present in the plasma membrane of spheroplasts.

When a suspension of spheroplasts was pulsed with 100 μl of H_2 -saturated incubation medium only a slight acidification of the suspending medium was observed (fig.1A). However, in the presence of the uncoupler FCCP (10 μM), which is known to increase proton permeability across the membrane [14], acidification of the external medium could be easily monitored. Using FCCP-mediated proton translocation across the plasma membrane, an increase in the proton content of the external medium proportional (at least for the small concentrations) to the amount of added H_2 (fig.1B) was measured. These results

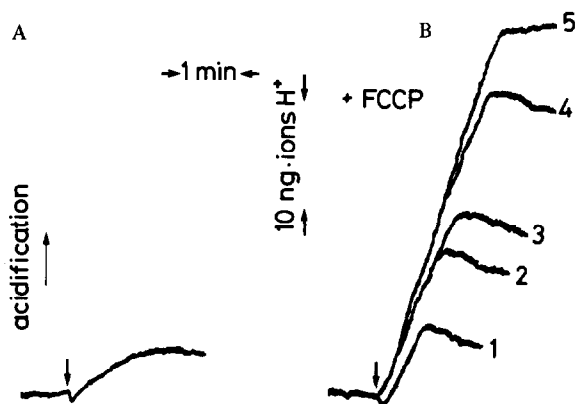


Fig.1. Proton release in the suspending medium upon H_2 -oxidation by benzylviologen in spheroplasts. Spheroplasts (10 mg protein) were equilibrated under a stream of argon in 0.3 M sucrose, 0.1 M KCl, 50 μM MES, 5 μM benzylviologen (pH 6.5) in 3 ml. The decrease in pH produced by injection of small quantities of H_2 -saturated medium (at the arrow) was followed in the absence (A) and the presence (B) of 10 μM FCCP. The H_2 -saturated medium used in (A) was 100 μl ; in (B) it was 10 μl , 20 μl , 30 μl , 50 μl and 100 μl for curves 1, 2, 3, 4 and 5, respectively.

indicated that the protons resulting from the oxidation of H_2 by benzylviologen were released in the cytoplasmic compartment of the spheroplasts.

3.2.2. Outward proton release with oxygen

On the other hand, when O_2 was added, instead of benzylviologen, as electron acceptor, protons were ejected (in the absence of FCCP) in the outward direction as is usually observed for respiration-driven proton translocation (fig.2).

The fact that oxidation of H_2 by benzylviologen cannot 'energize' the membrane and lead to energy-transduction was confirmed by measuring the level of phosphorylation of the endogenous adenine nucleotides. Cells of *P. denitrificans* (4.6 mg protein in 2 ml 10 mM HEPES, pH 7.5) were gassed for 10 min with either O_2 , N_2 or H_2 . When H_2 was used the cells were incubated for 3 min with benzylviologen (5 mM) as the electron acceptor [4]. Incubation was ended by adding 0.5 ml 5 N perchloric acid. After centrifugation of the proteins, the supernatant was neutralized with KOH and the adenine nucleotides estimated as in [13]. In aerobiosis, the adenine nucleotides were predominantly in the form of ATP. Under H_2 in presence of benzylviologen there was practically the same degree of dephosphorylation as under an atmosphere of nitrogen in the absence of electron acceptor.

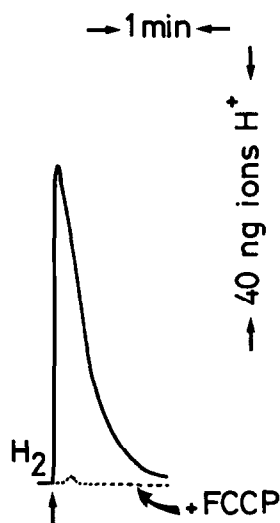


Fig.2. Outward proton ejection linked to H_2 oxidation by O_2 in spheroplasts. The spheroplasts (5 mg protein) in suspension in the medium of fig.1 were maintained under a stream of H_2 . At the arrow, air-saturated 0.1 M KCl solution (12 ng-atom O_2) was injected into the spheroplast suspension.

The energy charge [15] which was equal to 0.80 in aerobiosis dropped to 0.29 and 0.26 in anaerobiosis with H_2 and benzylviologen and with nitrogen, respectively.

The experiments reported in this section indicated that upon oxidation of H_2 -reduced hydrogenase by benzylviologen, protons were released in the inner, cytoplasmic compartment of the cell (fig.1) while when H_2 was being oxidized by O_2 , protons were ejected outwardly (fig.2). In this latter case the vectorial protons resulted from the functioning of the Mitchelian loops in the electron transport chain and contributed to the building of an electrochemical gradient of protons, negative inside, usable by the ATPase to make ATP. On the contrary, the protons released inside the cytoplasmic compartment should be considered as scalar protons, similar to those coming from NADH molecules upon their reoxidation, to be consumed in the cytoplasm when O_2 is reduced to form H_2O . While benzylviologen reacts directly with hydrogenase [4] it is not known whether or not a respiratory loop is involved when electron acceptors of higher redox potential such as fumarate are used. The involvement of a respiratory loop on the electron pathway from hydrogenase to fumarate might explain the difference in the results of [9] and ours.

3.3. Hydrogen-dependent outward proton release with sonicated membrane vesicles

The experiment done with spheroplasts (fig.1) was repeated with membrane vesicles (fig.3). In this latter case an immediate and direct release of protons in the suspending medium was observed upon pulse addition of small volumes of H_2 -saturated medium (fig.3A). When, on the other hand, FCCP was present there was a decrease in the amount of protons detected in the external medium due probably to a rapid FCCP-mediated proton equilibration on both sides of the membrane (fig.3B).

These results, showing that with inside-out vesicles the protons resulting from the oxidation of H_2 by benzylviologen were released in the external medium, confirmed the preceding ones obtained with spheroplasts. The two sets of results are clearly illustrated in fig.4. For the highest amounts of H_2 used there is no strict proportionality between the no. nmol H_2 injected and the ng-ions H^+ released in the medium (fig.4). This may result from H_2 losses since a current of argon was used to maintain anaerobiosis.

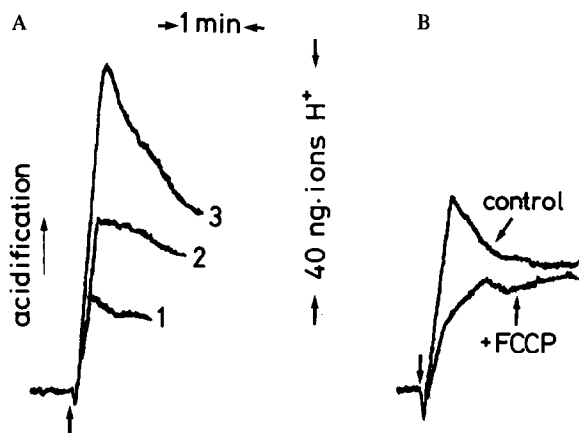


Fig.3. Proton release in the suspending medium upon H₂ oxidation by benzylviologen in sonicated membrane vesicles. Sonicated membrane vesicles (10 mg) were equilibrated under a stream of argon in 0.15 M KCl, and 5 μM benzylviologen in 3 ml. The decrease in pH produced by injection of small quantities of H₂-saturated incubation medium at the arrow was followed in the absence (A) and in presence (B) of 10 μM FCCP. The volume of H₂-saturated 0.15 M KCl solution added in (A) was 10 μl, 20 μl, 100 μl for curves 1, 2 and 3, respectively; in (B) it was 40 μl.

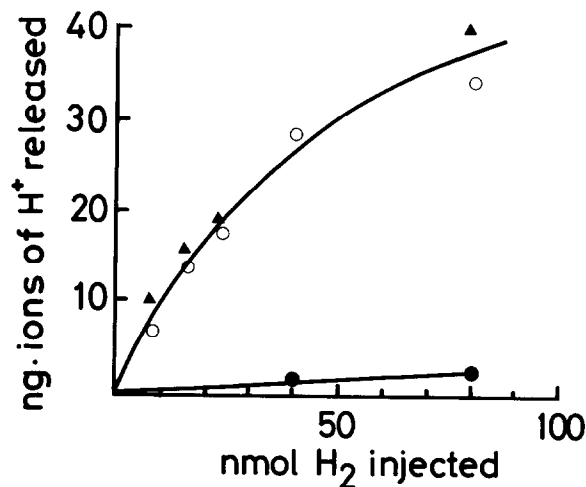


Fig.4. Proton release in the suspending medium in the presence of increasing H₂ concentrations: spheroplasts (●); spheroplasts + 10 μM FCCP (○); sonicated membrane vesicles (▲). Conditions as described in fig.1.

In conclusion, the activation of hydrogen by hydrogenase results in an inward release of protons in the cytoplasmic compartment of the cell. These protons act as scalar protons at the opposite of the respiration-driven vectorial protons outwardly ejected from the cell when electrons donated by H₂ are transferred through the loops of the electron transport chain to oxygen. Further work is necessary to identify a precise location of hydrogenase within the membrane and to determine quantitatively how many protons, from H₂, are released into the cytoplasm through the hydrogenase.

Acknowledgements

This work was supported by research grants from the 'Centre National de la Recherche Scientifique' (PIRDES) and the EEC Solar Energy Research and Development Program (grant ESF-538-78).

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